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Art. II.—*An Account of a Crown Rot of English Walnut Trees in Victoria.*

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(With Plate I.)

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Introduction.

A crown Canker or Root Rot disease of the Black Walnut (*Juglans Hindsii* Jepson) growing in Northern California became evident during the spring of 1922. R. E. and E. H. Smith (21), in a study of the disease, isolated a fungus which, on account of its cultural characteristics and the nature of its reproductive organs, was compared by them to *Phytophthora cactorum* (Cohn and Lebert) Schroeter.

A root disease of the English walnut (*J. regia* Linn.) has been established in the Bright district of Victoria for some years, of which one of the first symptoms is the occurrence of irregular black areas on the surface of the trunk, at, or just below, the level of the soil. Such regions ring hollow when knocked, and in them the dead bark is readily removed from the underlying wood cylinder. A quantity of a dark watery fluid may collect in a space formed by the separation of the stem tissues in the cambial zone, or this liquid may exude through cracks and appear on the surface as dark, gummy drops.

The leaves of a tree showing such symptoms will usually appear yellowish in colour, will fall early, and in time the whole tree will succumb to the effects of the disease.

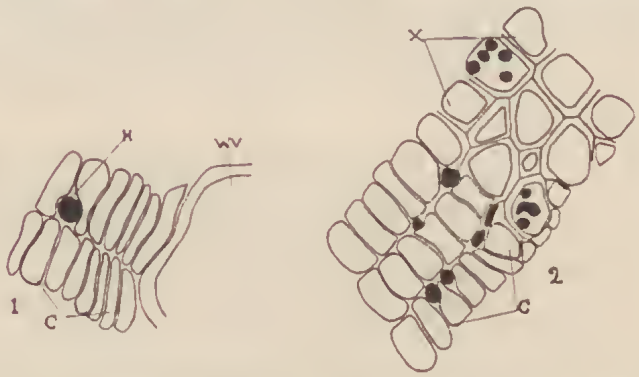
If the root system of a diseased tree be carefully examined, it will be found that many of the roots, of all sizes (those examined ranging from 7·5 cm.-1·5 cm. diam.), may be distinguished by the presence of irregular areas in which the bark has undergone a dark-brown discoloration, accompanied by the exudation of the brown liquid, as previously seen on the surface of the trunk. The darkening extends inwards as far as the wood, and involves a strip of the sap wood about 2 mm. wide. In later stages, here as in the stem, an almost complete separation takes place at the junction of the wood and the phloem, with the result that the bark is easily removed from the underlying wood cylinder. The discoloration is strictly limited, and usually includes only a small amount of the circumference of the root. In no case has an

entire girdling of the root been observed, although the presence of several darkened patches separated by healthy uncoloured bark is quite usual.

The Diseased Tissues.

Microscopic examination of the diseased areas has revealed in them the presence of non-septate fungal hyphae. These are clearly demonstrated in hand sections of the root which have been cleared in Lacto-Phenol solution, stained in 0·3% Cotton Blue in Lacto-Phenol and finally mounted in Lacto-Phenol according to the method adopted by Cook (7).

The hyphae of the invading fungus are only evident in the discoloured regions, none being present in the adjoining healthy tissues. The fungus is most abundantly found in the secondary phloem, adjacent to the cambial zone, where its hyphae occupy the intercellular spaces, and make their way between the cell walls of neighbouring cells. In the cambial region, intercellular

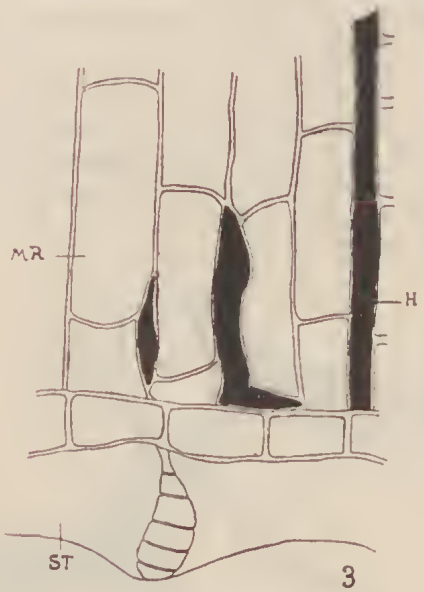


Figs. 1 and 2.—Transverse sections at junction between the cambium and wood, showing intra and intercellular hyphae. ×280.
h, hyphae; c, cambium; w.v, wood vessel; x, xylem.

hyphae occur abundantly (Fig. 2). The outer cortex of the root is usually free from infection, but hyphae have been observed between the starch-containing cells of the inner cortex. In the cortex, phloem and cambium, the general direction of growth is longitudinal, although many of the isodiametric, storage phloem and cortical parenchymatous cells are almost completely surrounded by branching hyphae. In the medullary rays, the very conspicuous and numerous hyphae follow a radial, intercellular course (Fig. 3); they can be traced both in transverse and radial longitudinal sections for considerable distances between the ray cells. In their passage in the radial direction they give off branches at intervals which, later, may themselves travel in the direction of the parent hypha.

A small strip of the youngest wood, though usually involved in the discoloration produced by the presence of the fungus, is

often free from fungal invasion. Occasionally, however, the hyphae enter both the young wood vessels and wood fibres, and in them pursue an intracellular and longitudinal course (Figs. 2 and 4). The hyphae, which branch freely and anastomose in the cavities of the fibres and vessels, have not been observed either in or



FIGS. 3 and 4.—3, Radial longitudinal section through secondary phloem. ! × 400. 4, Transverse section of sap wood showing hyphae in wood vessels and wood fibres. ×280. m.r. medullar ray; s.t. sieve tube; h. hyphae; w.f. wood fibres; w.v. wood vessels.

between the wood parenchyma cells. An entrance to the wood fibres and vessels is obtained by means of the bordered pits on their walls. The penetrating hypha undergoes considerable diminution in size as it enters the mouth of the pit, then becomes slightly enlarged in the region of the original middle lamella which has been dissolved during its passage, and finally narrows again as it leaves the pit to enter the cavity (Fig. 5).

The spread of the fungus in both a tangential and longitudinal direction appears to be limited by the very frequent development of cork tissue at the junction of the stained and unstained areas of the root. This self-limitation has been noted previously by Fawcett (11) in his study of "Pythiacystis Gummosis," and by Dufrenoy (9) during his investigations on the fungi causing "gummosis" of Citrus trees.

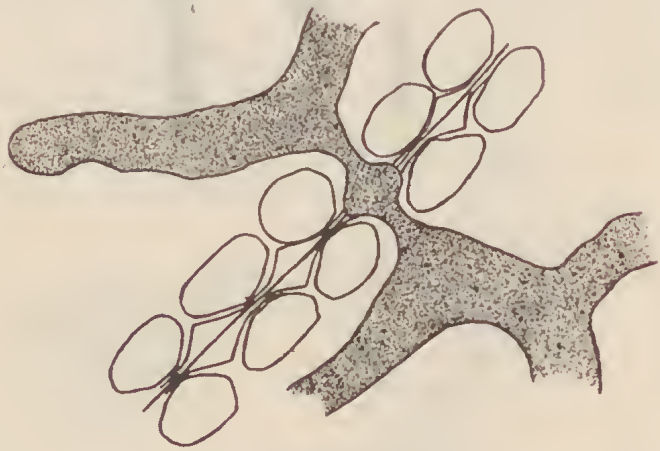


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Fig. 5.—The wood in longitudinal section showing the passage of hypha from one vessel to another. ×1000.

The mode of entry of the parasite into the host has not yet been determined. Its presence has been demonstrated in a small lateral rootlet 2 mm. in diameter, arising from a diseased root of 1·5 cm. diameter. There was no indication, however, in the example studied to suggest in which direction the fungus was travelling. No haustoria have been seen, and only the vegetative stage of the fungus has been met within the infected tissues of the walnut root.

The Fungus in Culture.

Many attempts to isolate the fungus in pure culture were unsuccessful. The method adopted was to remove with a sterilized razor the outermost layers of the bark, where saprophytic fungi were likely to be most abundant, and then to cut longitudinal sections of the tissue at the junction of wood and bark, including,

wherever possible, a portion of both the stained and healthy regions of the root. The sections thus obtained were placed in sterilized water, from which they were transferred to plates of 3% malt agar. One such culture, though bacterially contaminated, on 17th October, 1927, after five days at room temperature, showed a phycomycetous growth from the section of root tissue. A sub-culture was made on plain agar, from which it was possible to obtain pure cultures.

The fungus isolated grows readily on most of the media in general use, producing both a submerged and an aerial growth. The aerial mycelium is most abundantly developed on maize-meal, haricot bean, and oatmeal agar, and when young is composed of regular, sparingly-branched, thin-walled, non-septate hyphae, rich in granular protoplasm, and oil drops. Later, frequent septations occur, as well as a general thickening of the cell wall. This thickening, which is also met with in the submerged mycelium, is most evident in maize-meal cultures after a period of three weeks, when it occurs to such an extent as almost to obliterate the cavities of the filaments.

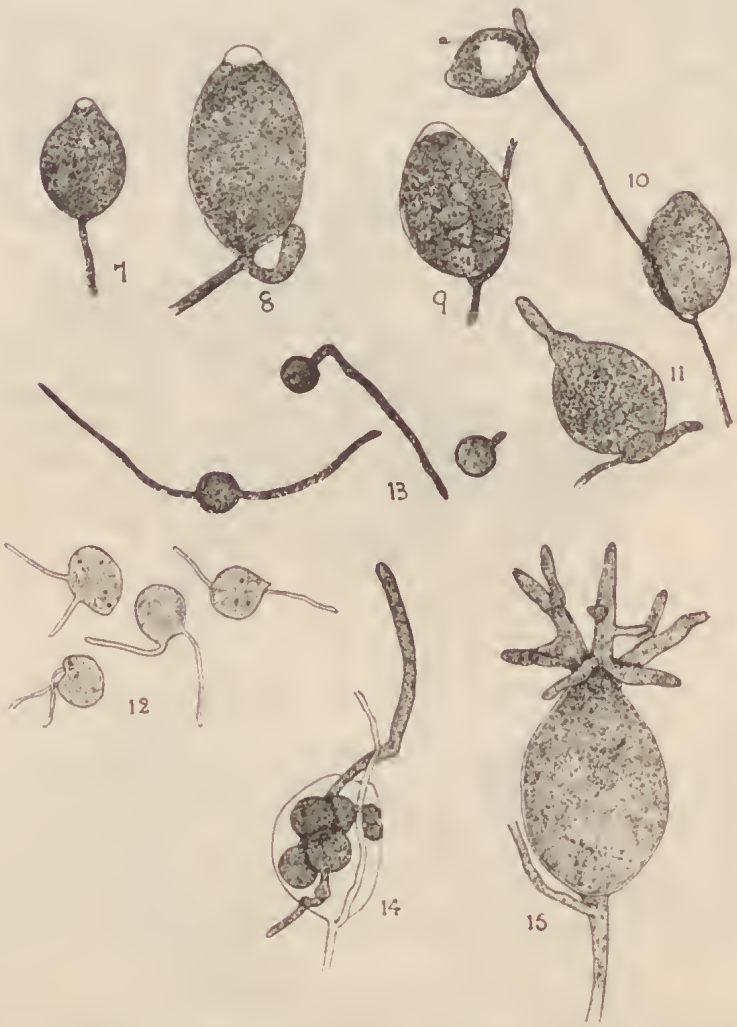
The submerged hyphae are uniform in diameter on oat agar, but in such media as malt agar, potato-dextrose agar, bean meal agar, and prune juice, become more or less irregularly swollen and gnarled (Fig. 6). With age, the finely granular hyphal contents are replaced by large drops of oil, which occur along the length of the hyphae.

Fig. 6.—*a*, Submerged hyphae from malt agar culture; *b*, Similar hyphae from potato-dextrose agar culture. Both ×400.

Conidia of the type characteristic of the genus *Phytophthora* have been developed when cultures of the fungus were made on

damp sterilized soil. The method adopted has been to obtain an active culture, by three or four days' growth in prune juice, which could then be transferred directly to a petri dish containing moist sterilized soil. After a week or longer, conidia were developed, though never in large numbers.

The conidia are ovate, occasionally elliptical, papillate, arising terminally on long unbranched conidiophores. The latter may



FIGS. 7-15.—7, Conidium with ratio of length to breadth of 1·49. ×400. 8, Conidium with ratio of length to breadth of 1·95. ×400. 9, Conidium from soil-agar culture after a period of two hours in water. ×400. 10, Conidiophore from soil-agar culture ×250. 11, Conidium 10*a* after 5 hours in water, germinating directly. ×400. 12, Zoospores killed with iodine. ×400. 13, Zoospores which have rounded off, germinating. ×400. 14, Conidium 6 hours after the escape of zoospores, showing the germination of unliberated zoospores in situ. ×400. 15, Conidium from 11 days' soil-culture, after 7 hours in water, showing the direct type of germination. ×400.

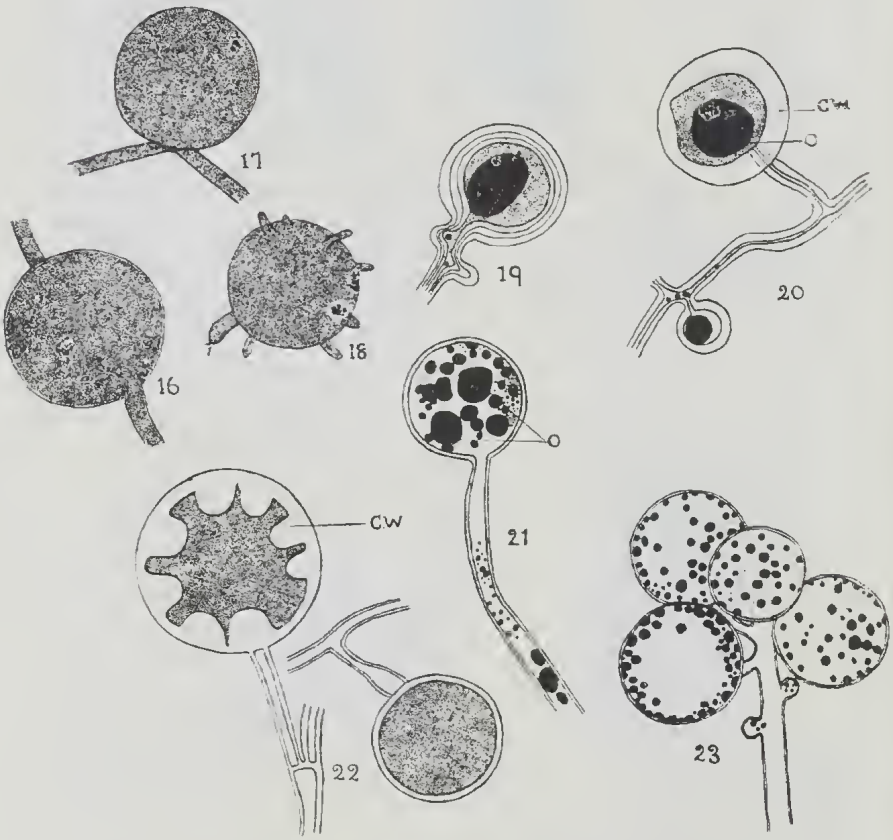
undergo further growth by which the conidium is pushed slightly to one side on a short pedicil, a sympodial arrangement thus being established (Figs. 8, 9, 10). The attachment of the conidium is always basal, no instance of a lateral position having been observed. The conidia vary very considerably both in size and shape, ranging from 30-82·5 μ in length, and 19·5-49·5 μ in breadth, the ratio of width to length varying from 1·11-2·00; while the average length of 114 conidia was 55·9μ, the average width of 114 conidia 36·6 μ and the ratio of length to width of 114 conidia was 1·50.

The germination of the conidium is readily observed by transferring portion of the mycelium to a drop of water on a glass slide. When immersed in a film of water, after a period of one to two hours at room temperature a variable number of zoospores are liberated. The conidium at first contains finely granular protoplasm (Figs. 7 and 8), but this soon is faintly delimited and the outlines of the individual zoospores become evident (Fig. 9). Just before maturity, a slight movement takes place in the contents of the conidium, after which the papilla gradually enlarges, being inflated as if by pressure from within. Meanwhile, some of the protoplasm flows into the papilla, the individuality of the zoospores appearing to be somewhat lost during the process. The papilla then bursts, and the zoospores separate from one another and rapidly swim away. The spores which have remained in the conidium now squeeze their way through the narrow opening of the sporangium, becoming dumbbell-shaped in the passage, and escape one by one. The zoospores are fusoid, biciliate bodies, and appear to be longitudinally grooved. Two vacuoles are usually present, and the cilia are generally of unequal length (Fig. 12). After a period of activity the zoospore settles down, rounds itself off, and germinates by means of one, or occasionally two, germ tubes (Fig. 13). The zoospore when rounded off and at rest has a diameter of from 12-13·5μ. The whole process from the time that the conidium was first observed to the time when the germ tube was quite evident was accomplished within eight hours on a cool day at room temperature.

Very often, one to several of the zoospores fail to leave the sporangium in spite of the activity of their movements and their close proximity to the exit point. These eventually become rounded off within the conidium and germinate there in situ, the germ tubes piercing the wall or else passing to the exterior through the papilla opening (Fig. 14).

If suitable conditions for zoospore formation do not obtain, the finely granular protoplasm of the conidium remains undivided, and one to several germ tubes arise from the region of the papilla, either as the direct outgrowth from this enlarged structure, or from the wall of the conidium around the papilla (Fig. 15).

Chlamydospores, or the "Resting Conidia" of Dastur (8), are the most characteristic organs met with in cultures of this fungus. Whereas the conidia are only produced when the fungus is grown on damp soil or soil agar, chlamydospores are abundantly developed on all the solid culture media used during this investigation. They occur on both the aerial and immersed mycelia, though most abundant in the subaerial and submerged portions of the growth. A typical chlamydospore is a spherical body borne either terminally, laterally, or intercalarly on a hypha (Figs. 16, 17, 22, 23). At first the cell wall is thin, and the contents are finely granular, only the shape and absence of a papilla distinguishing them from conidia (Figs. 17, 18). Later, the wall may



Figs. 16-23.—16 and 17, Intercalary chlamydospores from a soil culture. ×400. 18, Chlamydospore germinating after 9 hours in water at room temperature. ×400. 19, Thick-walled chlamydospore from 23 weeks' culture on maize-meal agar, showing successive deposition of cellulose layers. ×400. 20, Submerged mycelium with thick-walled chlamydospore from 20 weeks' culture on maize-meal agar. ×400. 21, Terminal chlamydospore on submerged mycelium of maize-meal culture. ×400. 22, Thick walled clamydospores from submerged mycelium of bean-meal culture. ×400. 23, A cluster of chamydospores from submerged mycelium of 6 weeks' potato-dextrose agar cufture. ×400. c.w. thickened cell-wall; o. oil globules.

become thickened by the deposition of layers of cellulose, as seen by the action of phosphoric acid and iodine, the degree of thickening depending both on the nature of the culture medium, and the age of the culture. On maize-meal agar, a maximum of thickening takes place; in some cases, especially in submerged chlamydospores, the diameter of the wall may measure as much as 9μ, when the cavity of the organ is almost obliterated, only a thin basal canal then connecting the reduced cavity with that of the supporting hypha (Figs. 19, 20). The successive layers of cell wall substance are clearly marked, and the width of the wall is even around its whole circumference (Fig. 19).

On bean-meal agar, many of the chlamydospores also become very thick-walled, but in this case the thickening is laid down in circular areas which project as blunt processes into the cavity of the spore. (Fig. 22).

In oatmeal, malt, and potato dextrose agars, though thickening of the vegetative hyphae may occur, the walls of the majority of chlamydospores are only slightly thickened.

All chlamydospores begin as thin-walled structures, and whether or not thickening of the wall takes place depends on external conditions. Their contents are dense, and at first finely granular, and this gives the appearance of slight coloration under a low magnification; but under a high power it is clearly evident that they are quite hyaline in character. This feature persists, for even in extreme age no yellowing is discernible. A central vacuole may sometimes be present. Later, small, regular globules which give a positive reaction with Sudan III fill the cavity of the spore, and in old specimens, especially the very thick-walled ones previously referred to, these run together to form fewer and larger oil globules (Figs. 20, 21, 23). Many of the chlamydospores in culture appear as empty sacs, devoid of contents, of which they have been drained apparently to supply a demand elsewhere.

Spherical chlamydospores are the most typical in form, but oval and irregular shapes are far from uncommon on the aerial and submerged mycelia. Their variation in diameter, however, is much greater, and in 225 spherical examples measured, ranged from 13·5 to 64·5μ with a mean of 35·96μ.

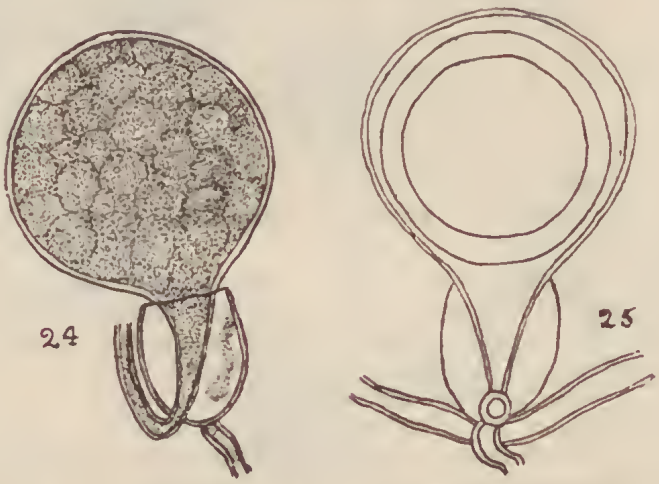
Germination of the chlamydospore takes place by means of a number of germ-tubes (Fig. 18); the abnormally thickened structures of bean and maize-meal cultures, however, have shown no power of further development.

Oogonia have not been observed in cultures on rich nutrient media, although carefully looked for; however, if these organs are only sparsely developed it is quite possible that they may have been overlooked. Oogonia are produced by this fungus, and were first seen in a plate culture prepared as follows:—A small portion of the mycelium was placed in prune juice for three days

at a temperature of 21° C. It was then washed in sterilized distilled water, and transferred to a plate of soil agar. When the plate was examined at the end of two weeks, the original inoculum showed a moderate number of oogonia with fully developed oospores. This method has been followed with success, but the sexual organs have always been restricted to the inoculum, and have never been observed on the scanty mycelial growth which spreads over the surface of the soil agar.

The oogonia are large, spherical bodies attached to the oogonial hypha by a well-defined, gradually-tapering stalk which, in most cases, lies within the basal antheridium. When young, the oogonium is thin-walled, colourless, and filled with numerous oil globules (Fig. 24; Plate I., Fig 3). Later, it becomes tinged with yellow, and as the oospore develops this coloration becomes intensified until a deep yellowish-brown colour results. The diameter of the oogonia varies considerably, and in 50 individuals ranged from 27 to 52·5μ with an average of 42·2μ.

The antheridium arises from a hypha which is distinct from that bearing the oogonium (Fig. 24), and usually entirely surrounds the basal region of the latter. At first the antheridium is thin-walled and hyaline, but later a slight thickening of the wall is accompanied by a coloration similar to that characteristic of the oogonium. The antheridium is very persistent, and varies in length from 18-25·5μ, and in breadth from 15-24μ.



Figs. 24, 25.—24. Antheridium and young oogonium. ×800. 25. Oogonium with mature oospore from the original inoculum of a 2 weeks' soil-agar culture. ×800.

The oospores are spherical, thick-walled, yellowish-brown bodies, which often do not quite fill the cavity of the oogonium (Fig. 25). The wall is 4·5μ in thickness, and the spore itself varies in diameter from 22·5 to 45·0μ with an average for 50 individuals of 35·3μ. The germination of the oospore has not been observed.

Culture Media.

The growth of the fungus isolated from diseased walnut roots, and above described, has been studied on several culture media.

Maize-meal Agar.—50 grs. of ground maize grains were steamed in 500 cc. of water for half hour, strained through wire gauze, 2% of agar added, and the whole made up to 500 cc. A thick white growth resulted on this medium, with aerial and submerged mycelia well developed. The aerial hyphae were even, sparingly branched, and at first non-septate. Many septa develop later, and the walls of the hyphae become very thick. No sporangia or oospores were produced. Chlamydospores were moderately developed on the aerial and very abundant on submerged mycelium, the wall of each becoming evenly and very considerably thickened over its whole surface.

Oatmeal Agar.—50 grams of crushed oats were steamed with 350 cc. water for half hour, strained through wire gauze, 2% agar added, with enough water to bring the quantity up to 500 cc., which material, after being brought to the boil, was tubed and autoclaved. In one week the culture had covered the surface of the plate, and the thick aerial growth reached a height of 1 cm. above the surface of the agar. The hyphae of the aerial mycelium were of constant diameter, sparingly branched, with numerous chlamydospores. The submerged and sub-aerial mycelia were also even with abundant chlamydospores arranged usually in clusters. The hyphae became septate, and some thick-walled; while many of the chlamydospores may become considerably thickened, the majority remain almost thin-walled even in old cultures. No sporangia or oospores were developed.

Bean-meal Agar.—50 grams of haricot beans were ground to a meal and added to 350 cc. water, steamed for half hour, strained through wire gauze, made up to 500 cc. with water and thickened with 2% agar. The aerial mycelium was thick and well-developed, reaching a height of 1·2 cm. above the surface of the agar. Its hyphae were regular, of even diameter, sparingly branched, thin-walled, continuous, at first quite sterile, but later developing a moderate number of chlamydospores. Submerged mycelium becomes irregularly swollen, and bears plentifully terminal chlamydospores, which may become very thick-walled by the deposition of rounded projections of cell wall substance. Oospores and conidia are not developed.

Potato Dextrose Agar.—Washed, but unpeeled, potatoes were cut into small cubes, 200 gms. were steamed in 1 litre of water for one hour, strained through cheese cloth, and made up to 1 litre with water to which 20 gms. of dextrose and 25 gms. agar were added. The aerial mycelium was well-developed, 1 cm. above surface of agar, though the growth was not as thick as that on maize, bean and oatmeal agars. The hyphae composing it were even and sparingly branched. The submerged mycelium was

irregularly swollen or gnarled. Chlamydospores were produced terminally or in little clusters or heads, and were thin or only slightly thick-walled, although the cells of the hyphae themselves frequently underwent considerable thickening. There were no conidia or sexual bodies.

Malt Agar.—3%. The surface growth attained a height of about 0·5 cm. above agar, but did not form a thick mat, the plate being transparent when held to the light. The surface hyphae were irregular in outline, and bore abundant chlamydospores in large groups. The submerged hyphae were very swollen and gnarled, with few perfect chlamydospores. The chlamydospores do not become very thick-walled, and oospores and conidia have not been observed.

Dilute Prune Juice.—Five prunes were cooked in 500 cc. water for half hour, strained through cotton wool, and filtrate made up to 500 cc. A quick and thick mycelial growth is obtained by planting in liquid prune juice. The submerged mycelium was thick, hyphae of which were very gnarled, with structures suggestive of imperfect chlamydospores, which, however, were probably merely swellings on the hyphae. A small amount of aerial mycelium was produced above the surface of the liquid, with even hyphae, and normal chlamydospores. Neither conidia nor oospores were developed.

Sterilized soil cultures have been considerably used, since on them conidia are always produced. Pieces of mycelium previously grown for at least three days in prune juice, and subsequently washed in sterilized water, were used as the inoculum. Growth was slow, and only a sparse mycelium produced. This, however, bore both conidia and chlamydospores, the latter being either intercalary or terminate on short lateral branches. No oogonia were noticed.

Soil Agar.—600 cc. water were added to 400 grams of Carrum sand and autoclaved at 125° C. for half hour. The extract was filtered through paper and the filtrate thickened with 1·5% agar. As in the case of soil cultures, here also the inoculum was previously grown for three or four days in dilute prune juice. The thin mycelium which gradually spread out to the edge of the plate was both submerged and closely applied to the surface of the agar, but no aerial growth was produced. Chlamydospores were only sparingly developed. The importance of this medium lies in the fact that oospores were developed in the original inoculum itself after growth of a week or more upon it. Conidia also were found in small numbers, but mainly on or near the inoculum.

Infection Experiments.

Only a few preliminary experiments have been made to test the capacity for growth of the fungus in living tissues.

1. The Castor Oil Plant (*Ricinus communis* L.).

Portion of the mycelium was placed on the upper surface of the lamina, near its junction with the petiole, of a young leaf of a seedling plant of the Castor Oil. Sterilized water was added in order to give sufficient moisture for the life and growth of the fungus, and the plant was covered with a bell jar; a non-treated plant being kept under identical conditions. After several weeks, one of the leaves of the infected plant turned brown, and assumed a water-soaked appearance, the apical region of the stem later exhibiting similar features, for a distance of about 5 cms. Portion of the stem was sterilized with 0·1% mercuric chloride solution, washed in sterilized distilled water, and sections made with a flamed razor were transferred to sterilized water and thence to oat agar plates. In a few days a non-septate mycelium had spread out from each of the sections, and the examination of the sub-cultures subsequently proved that a recovery of the original fungus had been made.

Sections of the stem were treated with lacto-phenol and cotton blue, when the presence of intercellular hyphae, particularly in the pith, was clearly distinguishable.

2. Fruits of the English Walnut.

Almost mature green walnuts were the only ones available. These were inoculated, under as sterile conditions as possible, with portions of the fungus, either by placing the mycelium directly upon the uninjured surface of the fruit, or by first cutting the surface with a sharp sterilized scalpel, and then placing the fungus over the point of injury. The infected fruits were placed in moist chambers, and kept at room temperature, controls being kept in every case.

In four days, a browning of the pericarp around the infection point was evident, in both types of inoculation, and this gradually extended until the whole of the mesocarp has assumed a dark-brown, water-soaked appearance. If the fruit was mature at the time of infection, splitting of the epicarp and mesocarp occurred, rotting of the tissues, however, continuing. If, as in several cases, the fruit was younger, the epicarp and mesocarp remained intact, turned dark brown, and a "dry rot" was established. The seed, also, became involved, the hyphae of the fungus which were seen on the surface of the endocarp being able to attack, penetrate and kill the young embryo. Hyphae also passed to the outer surface of the fruit, where they formed a white, fluffy, sterile growth.

Portions of the inner regions of the infected tissues were transferred to plates of 3% malt agar, and from these the fungus was readily recovered. An examination of sections of the brown, water-soaked tissues after treatment with lacto-phenol and cotton blue revealed the presence in them of numerous, branching, intercellular, non-septate hyphae.

3. Walnut Leaves.

Walnut leaves were placed in moist sterile petri dishes, and portions of the fungal mycelium were planted on the under surface, a drop of sterile water being added to each. Controls were similarly treated, but without the addition of the fungus. After four days, the leaf was blackened around the spot on which the fungus rested, the controls remaining unchanged.

4. Apple Fruits.

A number of ripe apples were used to test the pathogenicity of the fungus. The surface of the fruit was sterilized, and to this region the mycelium of the fungus was added, either with or without previous surface injury. The inoculated apples were kept in moist chambers at room temperature. Those fruits in which the surface layers were uninjured remained quite healthy until the end of the experiment, as did the controls. The injured apples, however, showed early evidence of fungal attack, since a brown rotting of the tissues soon appeared around the point of inoculation. This browning spread slowly around the circumference, the epidermal and deeper layers at the same time remaining intact. Finally, the entire apple became discoloured, and when cut open, showed that the whole flesh had been killed and browned. Examination of the tissues revealed the intercellular, non-septate hyphae of the fungus with a few small chlamydospores formed at intervals within them. The mycelium of the fungus was readily recovered from the dead tissues by ordinary methods of culture.

5. Walnut Seedlings.

Twelve to eighteen month old plants of the English Walnut were used for inoculation purposes, the fungus being planted either in the tissues of the stem near the ground level or on the surface of an exposed root.

The surface of the stem was treated with a 0·1% solution of mercuric chloride, washed with sterile water. A deep longitudinal incision was then made by means of a flamed scalpel, and into the pocket so formed, a three days old fungal colony, grown in prune juice, was carefully inserted. The wound was bound with moist, sterile cotton wool, and covered with oiled paper, the wrappings being kept moist for ten days with sterile water; after which time the coverings were removed. Controls were kept, and these remained healthy in every case.

In most instances infection became evident in ten days' time, and death followed within four weeks from the date of inoculation (Plate I., Fig. 1). In one case this was delayed for a period of three and a half months. The infected tissues assumed a brown coloration, and since the fungus travelled most rapidly in a vertical and only slowly in a tangential direction, this surface darkening appeared as a longitudinal strip of discoloured tissue

which extended from the wounded area. This longitudinal extension usually occurred both towards the stem apex and down through the hypocotyl to the root system. The fungus penetrated radially, and soon the tissue of the cortex, pith, phloem, and sometimes the wood were overrun by its intercellular hyphae. From such infected plants the fungus was reisolated without difficulty, and when examined showed all the cultural characters of the original inoculum.

Only a few preliminary experiments have been made, as yet, whereby infection has been established through root inoculation. In them, the terminal portion of the main root was exposed as carefully as possible, and wounded very slightly. The fungal culture was placed in position and bound to the root's surface with damp cotton wool. The wool was kept moist, and in three weeks' time the leaves wilted and the plant died. The tissues of the main, and secondary roots, hypocotyl, and stem to a distance of four inches from the point of inoculation were entirely browned, and showed in sections the presence of very numerous fungal hyphae.

The fungus appears to be far more widespread and luxuriant in its growth in infected seedlings than in the diseased tissues of adult trees. Rounded or oval swellings which cause considerable enlargement of the intercellular spaces are frequently present on the mycelium in the tissues of seedlings (Fig. 26). These closely resemble the chlamydospores produced by the fungus on nutrient media.

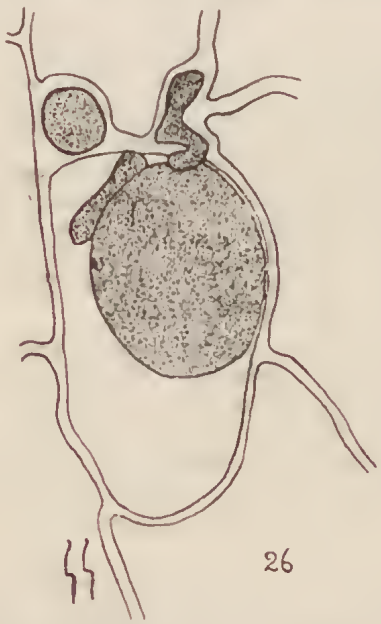


FIG. 26.—Intercellular hyphae in the outer cortex of an artificially infected Walnut seedling, showing a chlamydospore-like swelling, which has caused a considerable enlargement of the intercellular space. ×900.

Systematic Position of the Fungus.

From a study of the characters of the fungus isolated from the walnut roots, its affinity with members of the genus *Phytophthora* becomes clearly marked, and there seems no doubt that such is its generic position.

On account of the resemblances in the external symptoms of the disease with those characteristic of the "Gummosis" diseases of Citrus trees, a comparison with the organisms causing Brown Rot Gummosis, and Mal di Gomma Foot Rot (11) was naturally made.

Pythiacystis citrophthora Sm. & Sm. (20), the causal organism of Brown Rot Gummosis and Brown Rot of Lemon Fruits, has been recorded from Victoria by Brittlebank (2). In its typical form, the fungus only produces conidia, neither chlamydospores nor oogonia being developed. In this respect it differs markedly from the Walnut *Phytophthora* in which chlamydospores are a most characteristic cultural feature, and in which the production of oogonia can be definitely induced.

Pythiaceous forms have been isolated from several deciduous trees, including the Black Walnut by R. E. and E. H. Smith (21). Their strain "E" from the Walnut is of interest for comparison with the walnut *Phytophthora* under discussion. On fresh potato dextrose agar these authors report that the former yielded an abundance of oogonia and conidia, but no reference is made to the presence or absence of chlamydospores. The oospores average from 25-30μ in diameter, and the prominent antheridia are mostly paragynous, a position which has led to the suggested affinity with *Phytophthora cactorum* (Cohn and Lebert) Schroeter. Since oogonia and conidia are never produced on potato dextrose agar, while chlamydospores are abundant, and the oogonia are constantly amphigynous in the Victorian form, it would appear that the two organisms are distinct from one another. Several other strains which have been isolated from nursery trees of Almond, Pear, and Peach, have been described by these writers, but none of them agrees with the characters of the fungus under consideration.

The amphigynous nature of the oogonia suggests affinity with species of the Phaseoli group of *Phytophthora* as defined by Rosenbaum (18), which includes such forms as *P. parasitica* Dastur, *P. phaseoli* Thax., *P. infestans* (Mont.) de Bary, *P. erythroseptica* Peth., *P. arecae* Colem., *P. hibernalis* Carne, *P. Meadii* McRae, *P. colocasiae* Rac., *P. Faberi* Maulb., *P. cinnamomi* Rands., *P. cryptogea* Peth. and Laff., and *P. mexicana* Hot. and Hartge.

P. Faberi Maulb. (17) is distinguished from the Walnut *Phytophthora* in that it produces in cultures large numbers of more narrowly oval conidia which readily fall away from the coni-

diophore with a short refringent pedicel, and further sexual organs are only produced when it is grown in mixed cultures.

P. infestans (Mont.) de Bary need not be seriously considered since the conidiophores bear characteristic enlargements and smaller conidia, and the antheridia are often inconspicuous and not of constant occurrence.

In *P. phaseoli* Thaxt. (22) also the conidiophores undergo enlargement at the nodes, the sporangia are smaller, as also are the oospores than in the species under consideration, while the antheridia in the former are not persistent.

P. arecae Colem. (6), while showing some points of similarity to the Victorian form differs from it in the absence of chlamydospores. Similarly, *P. Meadii* (McRae, 14) only rarely develops chlamydospores in culture, and, as well, possesses more elongate conidia.

In *P. erythroseptica* Pethyb. (15) the most distinguishing feature is the inconspicuousness of the papilla of the conidium.

P. mexicana Hot. and Hartge (13) again has a more elongate conidium in which a very prominent papilla is evident, and chlamydospores are only sparingly produced.

P. hibernalis Carne (5) is distinct in that chlamydospores are never produced, while the conidia have a mean ratio of length to breadth of 2 or more, and fall from the conidiophore with a persistent pedicel.

One of the commonest forms of *Phytophthora* found in Victoria is *P. cryptogea* Pethyb. and Lafferty (16). This form has been studied by Brittlebank and Fish (3) who find that after the production of the sporangium the growth of the fungus is continued, a new sporangium being produced either in the old sporangium or a short distance from it. This feature has only once been seen in the Walnut species, while the oospores of the latter have a much higher average diameter.

P. colocasiae Rac. as described by Butler and Kulkarni (4) has elongated sporangia which are readily detached from the conidiophore with persistent pedicils. The oospores average 23μ in diameter, showing a difference of over 10μ from that of the walnut fungus.

P. cinnamomi Rands. (1) has oospores and oogonia which approximate closely to those described above for the walnut species, but the development of obpyriform sporangia without papillae clearly distinguishes the two forms.

The walnut fungus, on the whole, shows closest affinity with *P. parasitica* Dastur (8) although it differs very considerably from the size of the various spore types given in the original description of this species. Fawcett (10) has isolated from citrus trees suffering from Mal di Gomma foot rot a strain of *P. parasitica* which is identical with a fungus attacking the tomato and described as *P. terrestris* Sherbakoff (19). Godfrey (12)

has described a new variety of *P. parasitica* as the cause of foot rot of Rhubarb, while many other strains have been isolated from a variety of hosts.

Ashby (1), from a recent study of many of these strains, has given an emended and broader description of *P. parasitica*, and it is with this description that the characters of the fungus now being considered will be compared.

The conidia of the walnut *Phytophthora* agree in size and shape with those of *P. parasitica*, having a mean ratio of length to breadth of 1·5, and an average length of 55·9μ and breadth of 36·6μ. These bodies are not, however, produced in cultures on standard media, as is generally the case with the majority of the other strains. However, as in some of the latter their production is very scanty, it is thought that this cultural feature should not present a serious distinction.

Chlamydospores are formed in abundance by most *P. parasitica* strains, as they are in that from the walnut. In the latter, however, they remain hyaline throughout their life, never becoming coloured as has been described for other types.

The sexual organs are identical with those of the species considered, but they have not so far been found in ordinary cultures on such media as bean and oatmeal agars, as in other strains. The oogonia and oospores obtained by the method above described are large, and the average diameter higher than in the known strains, since the average diameter of the oospore is 35·3μ with a range from 22·5-45·0μ.

Ashby has now divided *P. parasitica* into two groups—

1. Microspora, in which the oospores have a mean diameter under 20μ with a range of 12-24μ.
2. Macrospora, with oospores of a mean diameter of over 20μ and a range of from 20 - ±35μ.

It will be seen, therefore, that the walnut strain must fall into the second sub-group of large-spored forms, and in doing so becomes separated from the organism causing Mal di Gomma foot rot of Citrus, i.e., *P. terrestris* Sherbakoff, which falls into the small-spored division.

P. parasitica has not been recorded as occurring in the State of Victoria, and it appears doubtful as to whether its presence in Australia has previously been noted. Mr. C. C. Brittlebank has very kindly shown me some unrecorded observations that he has made on an organism isolated by him on 25th February, 1925, from diseased Pine seedlings grown at the Forest Plantation, Creswick, Victoria, and assigned by him at that time to Dastur's species. It would seem then that his isolation marks the first discovery of this form in this State.

This work has been carried out in the Agricultural School of the University of Melbourne, and I wish to thank Professor Wadham both for his kindness in placing the facilities of his department at my disposal, and for his interest in and help during

the progress of the investigation. I am grateful to Mr. E. C. Dyason for arranging for the regular supply of diseased material from the groves at Wandiligong, and to Mr. A. O'Brien I am indebted for the photographic illustrations.

Summary.

1. The hyphae of a phycomycetous fungus have been demonstrated and traced in the inner region of the bark of diseased roots of the English Walnut (*Juglans regia*).

2. These hyphae are intercellular in secondary phloem and cambium; intracellular in the youngest layers of the wood.

3. A fungus showing characters typical of the genus *Phytophthora* has been isolated.

4. Conidia germinating either by means of zoospores, or more rarely directly by one or more germ tubes, are produced when active cultures are transferred to and grown on sterilized soil.

5. Conidia have an average length of 55·0μ, a breadth of 36·6μ, and an average ratio of length to breadth of 1·50.

6. Chlamydospores are very abundantly produced by the fungus when grown on maize-meal, bean-meal, oat-meal, agars, etc. They are hyaline, spherical bodies with a diameter ranging from 13·5 to 64·5μ, and showing a mean of 35·96μ.

7. Oogonia are produced when an active culture of the fungus is transferred to soil agar. They only occur among the hyphae of the original inoculum. They are yellowish-brown bodies with a diameter ranging from 27 to 52·5μ, the average being 42·2μ.

8. The antheridium completely surrounds the base of the oogonium. It is at first hyaline, later becoming brownish, and is persistent. The oospores are thick-walled, yellowish-brown, and vary in diameter from 22·5 to 45·0μ, an average of 50 individuals being 35·3μ.

9. The taxonomy of the fungus is discussed, and its affinity with *P. parasitica* Dastur, in the wide sense suggested by Ashby, is supported.

10. The fungus isolated and described has proved pathogenic to seedlings of *Juglans regia*, and from them has been reisolated in pure culture.

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